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THE INTESTINAL BACTERIAL FLORA OF RATS ON A DIET DEFICIENT IN FAT SOLUBLE VITAMIN A

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Intensive work of recent years appears to have established the specific nature of certain deficiency diseases of lower animals which can be made the subject of experimental investigation. Polyneuritis of fowls, scurvy of guinea-pigs, xerophthalmia and abnormality of calcification of rats and "leg weakness" of chicks are conditions whicn are apparently associated with the lack of essential and specific dietary constituents. The experimental diets used in such studies deviate quite widely from the normal diets of the various species of animals in a natural environment, and the deficient diet causes not merely the conditions mentioned, but also fails to support the growth of the animal. Although the test diet plus minute quantities of the specific substance or vitamin is adequate for growth, the question may be raised as to whether faulty assimilation due to abnormalities within the intestinal canal may have a part in the causation of the condition finally established.

McCarrison,[1] in his clinical and experimental study of the general metabolic disturbances associated with faulty diets, has ascribed a large share of the resulting condition to definite anatomic and physiologic abnormalities of the gastro-intestinal tract. It must be admitted, however, that the disturbances studied by McCarrison probably do not belong in the same category as the more striking and characteristic vitamin deficiency diseases mentioned. But even in some of the latter there has been some divergence of opinion as to the relative importance of vitamin deficiency and intestinal abnormality.

McCollum and Pitz[2] have claimed that experimental scurvy in the guinea-pig is not due to any vitamin deficiency but is rather the result of the absorption of toxic substances derived from putrefaction in the cecum caused by undue retention of feces. Any diet which does not possess such physical properties as will lead to the formation of bulky, easily eliminable feces, will produce scurvy in guinea-pigs. The earlier work of Jackson and Moody[3] supports this view in so far as intestinal abnormality is concerned, but ascribes greater importance to bacterial infection than to toxin absorption. They described coccus-like bodies in the lesions of scorbutic guinea-pigs and suc-

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[1] Jour. Am. Med. Assn., 1922, 78, p. 1.

[2] Jour. Biol. Chem., 1917, 31, p. 229.

[3] Jour. Infect. Dis., 1916, 19, p. 511.

ceeded in isolating a gram-positive diplococcus of low virulence which had a tendency to form chains and produced green on blood agar. Pure strains of this organism, when inoculated into the circulation of guinea-pigs living under ordinary conditions, gave rise in most instances to hemorrhagic and other lesions in bones, joints, muscles, lymphnodes and gums, the lesions being similar to those of guinea-pigs in which scurvy developed spontaneously as the result of faulty diets. They also observed that a cream diet and one of olive oil with milk led to fat constipation and early death. The latter finding McCollum and Pitz[2] believe supports the view that undue retention of feces is the primary factor in the development of scurvy in the guinea-pig.

Funk[4] and Torrey and Hess,[5] on the other hand, are of the opinion that scurvy is a deficiency disease due to the absence of the antiscorbutic vitamin. Torrey and Hess[5] investigated the relation of putrefactive bacterial activity in the intestinal tract of scorbutic guinea-pigs. They made a study of the intestinal flora of guinea-pigs on normal diet, on a diet which produced scurvy, and on a diet which cured this disorder. They found that there was no change in the flora at any time, although the scorbutic symptoms disappeared on a diet which supplied the supposed deficiency.

In the case of "leg weakness" of chicks, there has also been some difference of opinion. Osborne and Mendel[6] had claimed that in this condition the lack of suitable roughage in the ration is an important factor, the roughage being necessary for the formation of easily eliminable feces. Hart, Halpin and Steenbock[7] confirmed these findings, but later[8] found that the addition of cod-liver oil protects chicks against the development of "leg weakness," and that the disease develops if this vitamin-containing accessory is omitted even if suitable roughage is supplied.

That dietary changes may cause alterations in the intestinal bacterial flora has been established by Rettger and his co-workers.[9] Such alterations are especially easily produced in the rat, the classical test animal in studies of the fat soluble vitamin A. In connection with some other work in which rats were being used for a study of this accessory factor, it seemed important to investigate the changes which might occur in the intestinal flora and in the physical characters of the feces in order that possible intestinal factors might be properly evaluated in the interpretation of the general constitutional changes which occurred in the animals.

White rats were used. The feces were collected according to the manner described by Hull and Rettger.[10] The rats were held by the tail and rubbed on the back at the base of the tail. The feces were collected directly into sterile test-tubes and weighed. They were then emulsified in sterile salt solution in the ratio of 1 gm. of feces to 10 c c of solution. From this primary emulsion, dilutions of 1 : 1,000, 1 : 10,000, and 1 : 100,000 were made and plated. From the original emulsion smears were prepared for staining and microscopic examination. The water content of the diet varied so little that this factor was believed to be without effect on the bacterial counts.

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[4] Biol. Chem. Bull., 1915, 4, p. 304.
[5] Proc. Soc. Exper. Biol. and Med., 1918, 15, p. 74.
[6] Jour. Biol. Chem., 1918, 33, p. 433.
[7] Ibid., 1920, 43, p. 421.
[8] Ibid., 1922, 52, p. 378.
[9] A Treatise on the Transformation of the Intestinal Flora, 1921.
[10] Jour. Bacteriol., 1917, 2, p. 47.

The rats were fed on a diet known to cause vitamin A deficiency diseases. This consisted of casein 18%, dextrin 72%, salts 4% and yeast 6%. In addition, the control rats received from 2 to 3 drops of cod-liver oil. This is considered, and was found to be, an adequate normal diet in regard to vitamin requirement. Whenever the rats on the deficient diet became ill, they were given from 2 to 4 drops of a cottonseed oil solution of an unsaponifiable residue from cod-liver oil which was partly freed from cholesterol. This immediately caused the disappearance of those manifestations which had been brought about by the deficient diet. The fecal material of the control rats, of the rats on a diet deficient in vitamin-A, and of the treated rats on the diet with the unsaponifiable residue was used for comparative study.

The following mediums were used, being inoculated at 37 C. and colony counts made at the end of 48 hours:

Beef liver glucose agar, with an initial reaction of PH 6.8. On this the characteristic fluffy colonies of B. acidophilus were produced.

Endo's agar was used for the determination of the proportion of bacteria able to ferment lactose; these were considered as being probably B. coli.

Glucose and saccharose agar plates with Andrade indicator permitted the estimation of the number of bacteria which fermented these two sugars.

Blood agar plates were used for counting streptococcus colonies.

Lead acetate agar gave quantitative information as to the number of bacteria able to form hydrogen sulphide.

Gram stains were made of smears of the fecal emulsions and a differential count was made of the gram-positive rods, gram-negative rods, gram-positive cocci and gram-negative cocci. The successive counts on the individual rats were averaged, and from these average counts for all the rats on the control diet and for those on the deficient diet an average for each group of rats was obtained.

On the control diet the percentage of gram-positive rods varied from 11.8 to 19.6 in the individual animals, with a group average of 16.8; in the animals on deficient diet the percentage varied from 11.3

to 24.8, with an average of 17.4. In the control group the percentage of gram-negative rods varied from 66.8 to 72.7, with an average of 69.8; in the experimental group from 62.3 to 75.3, with an average of 68.9. Gram-positive cocci varied from 7.5 to 15.5% in the control animals, with an average of 12.1%, and from 7.7 to 17.8% in the rats on deficient diet, the average being 11.3%. Gram-negative cocci varied from 0.5 to 2.7% in the control group, with an average of 1.3%; in the other group the individual variations were from 0.8 to 3.7%, the average being 2.4% (table 1). The deficient diet, therefore, caused no noteworthy changes in the proportion of the 4 groups of bacteria based on gram stain and morphology. Streptococci disappeared entirely from the flora of 7 of the rats on low vitamin diet. In 4 of these animals the B. acidophilus percentage went up. In the other 3 the percentage of gram-negative rods rose, or both gram-negtive and gram-positive rods increased proportionately. There was no change in the differential count when the vitamin-containing residue was added to the diet, although all symptoms of sickness disappeared from the rats when this addition was made.

TABLE 1

DIFFERENTIAL FECAL BACTERIAL COUNTS OF RATS ON NORMAL DIET AND ON DIET LACKING VITAMIN-A

Normal Diet					Diet Deficient in Vitamin-A				
Rat No.	Gram-Positive Rods %	Gram-Negative Rods %	Gram-Positive Cocci %	Gram-Negative Cocci %	Rat No.	Gram-Positive Rods %	Gram-Negative Rods %	Gram-Positive Cocci %	Gram-Negative Cocci %
102	19.6	66.8	13.2	0.5	106	11.3	75.3	12.7	0.8
103	19.0	72.7	7.5	0.8	110	13.4	74.5	10.2	2.0
104	11.8	70.0	15.5	2.7	112	18.3	68.2	10.7	2.9
					201	18.1	72.3	7.7	1.9
					204	24.8	62.7	8.8	3.7
					205	17.1	62.3	17.8	2.8
					206	18.7	67.3	11.2	2.8
Average....	16.8	69.8	12.1	1.3		17.4	68.9	11.3	2.4

The bacterial count per cubic centimeter of the original emulsions differed to a marked degree as the rats were kept on the diet lacking in fat soluble A. The feces became dry and hard as the disease became more advanced. With this change in the physical character of the feces, the bacterial count decreased. Within 24 to 48 hours after the unsaponifiable residue had been given, the feces became moist and soft and the bacterial count greatly increased (table 2). Whether this change, which was striking and the only one noted, was due to an absence from the intestinal canal of accessory factors perhaps necessary for bacterial growth, to the low food intake of the animals in their ill condition, or to a nutritional condition of the host animal so poor that it could not support even a normal intestinal flora, is left undecided. That it is a fundamental factor in the causation of the condition characteristic of vitamin A deficiency is doubtful. The quantitative alteration in the intestinal flora may be a secondary factor in bringing about the disturbed general metabolism of rats on a deficient diet, by altering digestion within and assimilation from the intestinal tract. Even this change in the number of bacteria is not a constant one. In the group of 5 rats included in table 2, the low bacterial count persisted in 105 and 207, even after the addition of vitamin-A, although the evidences of illness disappeared. In the case of rats 112 and 201 (table 2), there was a sudden great increase in the fecal bacterial content on the day that the vitamin was first administered, the increased count being evident in fecal samples taken 2 hours after the administration of the vitamin. No explanation of such a sudden increase is attempted. Whenever the fecal bacteria increased after the administration of vitamin-A the increase was

TABLE 2

FECAL BACTERIA PER C C BEFORE AND AFTER ADDITION OF VITAMIN-A TO DIET

Rat No.	Date	Glucose Fermenters	Streptococci	B. acidophilus	Remarks
202	4/11	70,000	200,000	230,000	Vitamin-A given May 23, 14, 25 and 26
	5/26	4,000,000	None	100,000	
	5/31	15,000,000	None	100,000	
	6/ 7	2,000,000	None	100,000	
206	4/11	700,000	60,000	160,000	Vitamin-A given June 1, 2, 3, and 5
	5/11	900,000	50,000	140,000	
	5/18	200,000	4,000	39,000	
	6/ 5	1,400,000	None	120,000	
112	4/17	63,000	10,000	10,000	Vitamin-A given May 15, 16, 17, 19, 20, 22, 23, 24, 25, 26, 27, 29, 31, June 1, 2, 3, 5, 6, 9
	4/20	700,000	30,000	15,000	
	5/15*	2,000.000	45,000	8,000	
	5/24	1,000,000	30,000	120,000	
	5/25	4,000,000	200,000	200,000	
	5/26	3,500,000	50,000	200,000	
	6/ 1	4,000,000	20,000	150,000	
201	4/11	160,000	21,000	45,000	Vitamin-A given May 25, 26 and 27
	4/20	350,000	5,000	250,000	
	5/ 5	2,000,000	3,000	320,000	
	5/18	130,000	None	90,000	
	5/25*	20,000,000	860,000	130,000	
105	4/10	200,000	360,000	350,000	Vitamin-A given May 24, 25, 26, 27, 29, 31, June 1, 2, 3, 5, 6, 7, 8, 9
	6/ 5	49,000	None	11,000	
	6/ 8	60,000	12,000	None	
207	4/14	64,000	None	10,000	Vitamin-A given May 14, 15 and 16
	5/18	37,000	None	8,000	

* First dose of vitamin administered at 1 p. m., fecal specimen taken at 3 p. m.
All the rats of this group were placed on deficient diet March 24.

chiefly of the glucose-fermenting bacteria, streptococci and B. acidophilus remaining unchanged or undergoing inconstant changes in number.

The glucose, lactose and saccharose mediums showed no differences associated with the change of diet, except increase or decrease as the total number of bacteria changed.

The ratio of hydrogen sulphide forming bacteria to total bacteria, as determined by lead acetate agar plates, varied from 1: 3.4 to 1: 13.8 in the control group, the average being 1:8; the total number of sulphide-forming bacteria was fairly constant, but there was considerable difference in the total bacterial counts of the individual animals. In the rats on low vitamin diet, both the hydrogen sulphide formers and the total bacteria were more uniform; the ratio of the former to the latter varied from 1:6 to 1:8.3, average 1:7.2 (table 3). The changes noted are so slight as to be without significance.

TABLE 3

HYDROGEN SULPHIDE FORMING BACTERIA OF RATS ON NORMAL DIET AND ON DIET DEFICIENT IN VITAMIN-A

Normal Diet				Diet Deficient in Vitamin-A			
Rat No.	No. of Sulphide Formers per C c	Total Bacteria per C c	Ratio	Rat No.	No. of Sulphide Formers per C c	Total Bacteria per C c	Ratio
102	12,000	40,750	1: 3.4	106	6,666	44,666	1:6.7
103	7,700	57,000	1: 7.4	110	8,600	71,200	1:8.3
104	10,000	138,500	1:13.8	112	5,714	42,428	1:7.4
				201	5,600 .	40,800	1:7.3
				206	7,750	46,500	1:6
Average	9,900	78,750	1:8.0		6,866	49,119	1:7.2

SUMMARY

In a study of the fecal bacteria of a group of rats on a diet so deficient in fat-soluble vitamin A as to result in xerophthalmia and other conditions characteristic of this deficiency, as compared with a group on an adequate control diet, no change occurred in the relative proportions of gram-negative rods, gram-positive rods, gram-positive cocci, and gram-negative cocci, as determined by smears made from standard emulsions of the freshly collected feces.

On the deficient diet the feces of the majority of the animals became dry and hard and the total number of viable bacteria was greatly decreased. When these animals were given fat soluble A in an amount sufficient to cause a disappearance of lesions, the feces became moist and soft and the total number of bacteria increased strikingly.

In most of the animals on the deficient diet, streptococci disappeared completely. This may have been due to the unfavorable conditions brought about in the intestinal canal by the inadequate diet, the less resistant streptococci disappearing while the more hardy species merely decreased greatly in number.

There was no change in the proportions of bacteria which fermented glucose, lactose and saccharose.

The proportion of hydrogen sulphide forming bacteria remained constant.